Biosynthesis of ω-cyclohexyl fatty acids in *Alicyclobacillus acidocaldarius*: the stereochemistry of the initial 1,4-conjugate elimination

Sandeep Handa and Heinz G. Floss*

Department of Chemistry, Box 351700, University of Washington, Seattle, Washington 98195-1700, USA

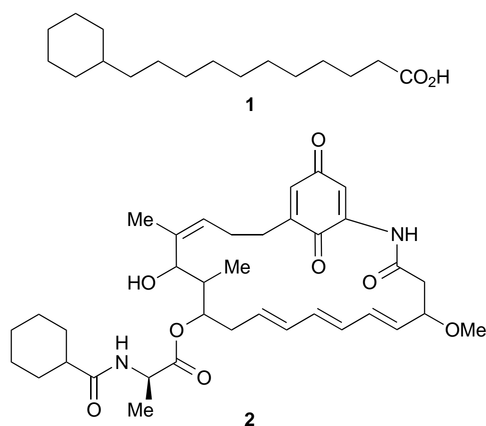
The 1,4-conjugate elimination of H_2O from shikimic acid 3, the initial step in the biosynthesis of ω-cyclohexyl fatty acids by the bacterium *Alicyclobacillus acidocaldarius*, has been shown to occur with an overall *anti* stereochemistry *via* loss of the *pro*-6*R* hydrogen of 3.

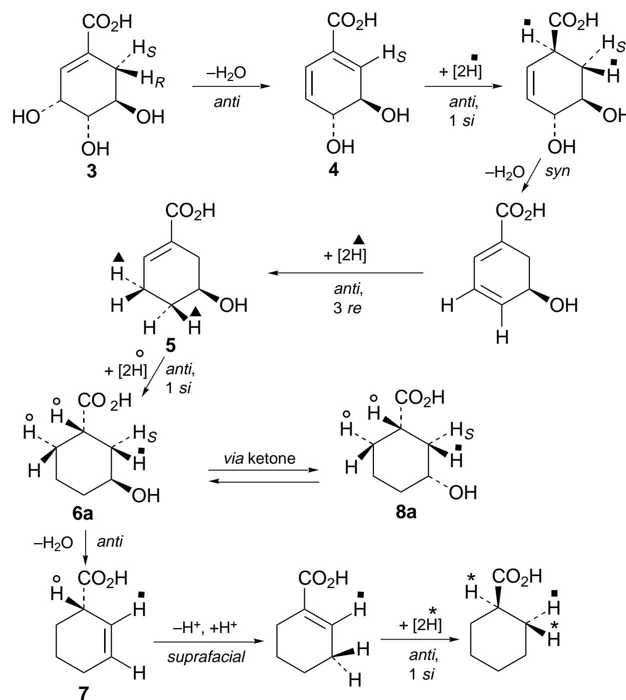
The thermoacidophilic bacterium *Alicyclobacillus acidocaldarius* (formerly *Bacillus acidocaldarius*) produces a series of ω-cyclohexyl fatty acids of which ω-cyclohexylundecanoic acid **1** is the most abundant.[1] It has been suggested that these unusual fatty acids have special physiological importance for the cells at the high temperatures and low pH at which they grow.[2] ω-Cyclohexyl fatty acids have also been isolated from the thermoacidophilic *A. acidoterrestis*[3] and the mesophilic *Curtobacterium pusillum*[4] and in all cases they are believed to arise *via* fatty acid biosynthesis from a cyclohexanecarboxylic acid starter unit (presumably as its coenzyme A thioester). This fully reduced cyclohexanecarboxylate moiety is relatively rare in nature although it is found attached, *via* a **d**-alanine unit, to the macrocycle of the ansamycin antibiotic ansatrienin A **2** produced by *Streptomyces collinus*.[5]

The biosynthesis of the cyclohexanecarboxylic acid moiety has been studied in *A. acidocaldarius*[6] and *S. collinus*[7] and it has been shown to arise from shikimic acid **3** *via* a series of dehydrations and double bond reductions such that no intermediate is ever aromatic. Previous studies have allowed us to delineate all the steps in the biosynthetic conversion and to establish the stereochemical course for all but one of the metabolic transformations. The biosynthetic pathway, which is common to both organisms, is shown in Scheme 1.

To date, however, the stereochemistry of the initial 1,4-conjugate elimination, resulting in the conversion of shikimic acid **3** to (3*R*,4*R*)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid **4** by loss of an unactivated C-6 proton and the C-3 hydroxy group of **3**, has remained elusive. This is primarily due to the fact that both the C-6 protons of **3** are lost in the overall conversion to cyclohexanecarboxylic acid, thus obscuring the stereochemistry of the initial proton loss. This problem was overcome by the isolation of a blocked mutant of *A. acidocaldarius*[8] (designated as mutant 2) which is unable to convert the *trans*-3-hydroxycyclohexanecarboxylic acid intermediate **6a** to cyclohex-2-enecarboxylic acid **7**. This results in the accumulation of **6a**, isolated from the culture medium as its methyl ester **6b**,[6] and lower levels of its C-3 epimer *cis*-3-hydroxycyclohexanecarboxylic acid **8a** (again isolated as the corresponding methyl ester **8b**), postulated to arise from **6a** by a side reaction involving oxidation to the ketone and reduction with opposite stereochemistry.[6] Since both **6a** and **8a** contain the shikimate C-6 proton retained in the initial 1,4-conjugate elimination step, the *A. acidocaldarius* mutant 2 provided a system in which to investigate the stereochemistry of this elimination.

In order to synthesize the stereospecifically deuteriated (6*R*)-(−)-[6-$^2H_1$]shikimic acid **3a** required for these investigations, we decided to employ the route of Fleet,[9] as previously adapted by Zamir *et al.*[10] (Scheme 2). This adaptation allows for the stereospecific labelling to be introduced *via* enzymic reduction of the intermediate aldehyde **9a** (prepared from **d**-mannose) using horse liver alcohol dehydrogenase coupled to an NAD^+ recycling system employing [$^2H_6$]ethanol to supply the deuteride reducing equivalents. The intermediate alcohol **10a** thus produced was then taken through to **3a** as previously described.[9] We were also able use this strategy to synthesise (6*S*)-(−)-[6-$^2H_1$]shikimic acid **3b** *via* the corresponding enzymic reduction of the deuteriated aldehyde **9b** (prepared from **9a** by

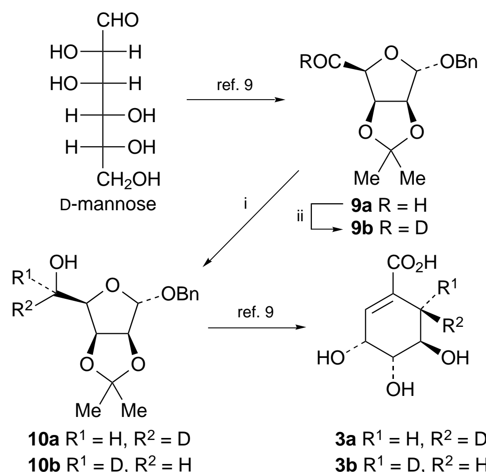




Scheme 1

an oxidation–reduction sequence) using unlabelled ethanol to supply the reducing equivalents.

With both specifically deuteriated shikimates **3a** and **3b** in hand feeding experiments were carried out with cultures of *A. acidocaldarius* mutant 2. The two accumulated intermediates **6a** and **8a** were isolated as their methyl esters **6b** and **8b**, employing similar conditions and experimental protocols to those previously described.[6] The isolated compounds were subsequently analysed by ^{2}H NMR and two of the resulting spectra are shown in Fig. 1.



Scheme 2 *Reagents*: i, Horse liver alcohol dehydrogenase, NAD$^+$, C_2D_5OD (for **9a**), C_2H_5OH (for **9b**); ii, NaO_2Cl, 2-methylbut-2-ene, Bu^{t}OH, H_2O, then CH_2N_2, Et_2O, then $LiAlD_4$, THF, then Me_2SO, $(COCl)_2$, $Pr^i{}_2EtN$, CH_2Cl_2

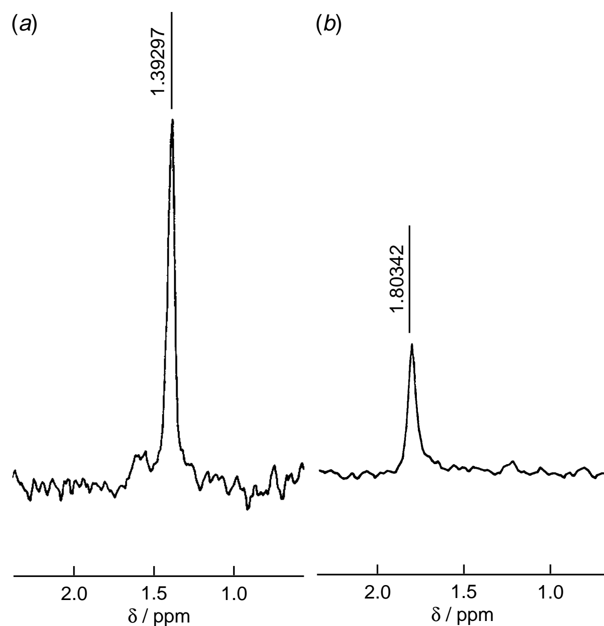


Fig. 1 Partial 46.07 MHz ^{2}H NMR spectra of (*a*) **8b** and (*b*) **6b** from the incorporation of (6*S*)-(−)-[6-$^2H_1$]shikimic acid **3b**. The spectra of **6b** and **8b** obtained from (6*R*)-(−)-[6-$^2H_1$]shikimic acid **3a** showed no signals in this region.

Previous studies[6] had already established that the proton retained in the initial 1,4-conjugate elimination from **3** occupies the *pro*-2*S* position in the acids **6a** and **8a** accumulated by the mutant organism. Furthermore, ^{1}H NMR spectra of the corresponding methyl esters **6b** and **8b**, while not allowing definite identification of the signal attributable to this proton, had shown it to lie within a complex set of signals between δ 1.45–1.85 for the *trans*-isomer **6b** and between δ 1.15–1.45 or 1.78–1.96 for the *cis*-isomer **8b**.

Inspection of the ^{2}H NMR spectra of **6b** and **8b** isolated from the feeding experiment with (6*S*)-(−)-[6-$^2H_1$]shikimic acid **3b** (Fig. 1) clearly shows that the deuterium label has been retained, giving rise to signals at δ 1.80 and 1.39 for **6b** and **8b**, respectively. The ^{2}H NMR spectra of **6b** and **8b** isolated from the experiment with (6*R*)-(−)-[6-$^2H_1$]shikimic acid **3a** showed no discernible signals even after prolonged data accumulation.

Thus the results clearly indicate that it is the *pro*-6*R* hydrogen of shikimate that is lost in the initial 1,4-elimination step in the biosynthesis of cyclohexanecarboxylic acid by *A. acidocaldarius*. The elimination therefore occurs by an overall *anti* mechanism and as such mirrors exactly the analogous 1,4-elimination found in the 'normal' shikimate pathway involving the formation of chorismate from 5-enolpyruvylshikimate 3-phosphate.[11] While previous experiments[12] with *A. acidocaldarius* have shown that chorismate is not an intermediate on the biosynthetic pathway to cyclohexanecarboxylic acid, confirming divergence from the standard shikimate pathway prior to this stage, this new finding suggests that there may be a strong evolutionary relationship between the two metabolic processes, which share the feature of abstraction of an unactivated hydrogen. Additionally it removes the final stereochemical ambiguity of the biosynthetic pathway.

Further work will explore mechanistic aspects of this elimination (*e.g.* whether the hydroxy group is activated by phosphorylation prior to elimination, as is the case in the formation of chorismate) and will examine the stereochemistry of similar reactions in analogous biosynthetic pathways.

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